Review

Signal recognition and transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants

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ABSTRACT – Plant disease resistance is the result of an innate host defense mechanism, which relies on the ability of the plant to recognize pathogen invasion and to efficiently mount defense responses. In tomato, resistance to the pathogen Pseudomonas syringae is mediated by the specific interaction between the plant serine/threonine kinase Pto and the bacterial protein AvrPto. This article reviews molecular and biochemical properties that confer to Pto the capability to function as an intracellular receptor and to activate a signaling cascade leading to the induction of defense responses. © 2000 Éditions scientifiques et médicales Elsevier SAS

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1. Plant disease resistance: a case of innate immunity

The concept of innate immunity in animals refers to the first line of host defense responses that limit infection in the early hours after exposure to microorganisms [1]. In plants challenged by pathogens, the early and rapid activation of an array of defense responses leading to disease resistance represents a case of innate immunity. Among the remarkable responses associated with resistance to pathogen invasion in plants, is rapid localized cell death at the site of infection, termed the hypersensitive response (HR), which is thought to limit pathogen growth and spreading throughout the infected plant. Additional early and local molecular events associated with disease resistance include the production of reactive oxygen species and nitric oxide, transient opening of ion channels, cell wall fortifications, production of antimicrobial phytoalexins, and synthesis of pathogenesis-related (PR) proteins with antifungal and antibacterial properties, such as glucanases and chitinases [2, 3].

In recent years genetic analysis combined with molecular and biochemical approaches have started to shed light on the pathways that mediate disease resistance in plants [4, 5], and revealed interesting parallels to mechanisms of innate immunity in animals (figure 1). In many plant–pathogen interactions, the rapid activation of defense responses is mediated by a specific recognition event involving an avirulence (avr) gene in the pathogen and the corresponding resistance (R) gene in the plant. Pathogen genes, which often contribute to virulence in susceptible plants, are referred to as avirulence genes if their products may be recognized and elicit defense responses in resistant plants. R genes encode intracellular or transmembrane proteins, which in a receptor–ligand interaction specifically recognize avr gene products delivered directly inside the plant cell or to intercellular spaces. Many R genes have been identified to date and grouped in different classes according to their structural characteristics [4]. Interestingly, two distinct classes of R gene products share similarities with proteins from mammals and insects that are involved in mechanisms of innate immunity (figure 1). One of these classes includes putative cytoplasmic proteins that, in addition to having similar nucleotide binding sites and leucine-rich repeats, share a domain with intriguing similarity to the cytoplasmic domain of the mammalian interleukin-1 (IL-1R) and the Drosophila Toll receptors. IL-1R plays a central role in the immune and inflammatory responses in mammals by recognizing the IL-1 cytokine and activating a signaling pathway, which results in the translocation of the transcription factor NF-κB to the cell nucleus [6]. By an analogous chain of events involving similar components, Toll in Drosophila mediates a signaling pathway required for the determination of dorsoventral polarity during development, and for the production of peptides with antifungal activities during pathogen infection [1, 7].

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A second class of plant resistance proteins with homology to components of innate immunity signaling pathways is represented by the tomato Pto, which confers resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* [8]. Pto is a serine/threonine kinase that shares significant similarities in the kinase catalytic domain with the human IL-1R-associated kinase (IRAK) and the *Drosophila* Pelle which are downstream components of the IL-1R and Toll receptors [6, 7].

More general similarities between mechanisms of innate immunity in mammals, insects and plants include the involvement in different steps of the pathways of reversible protein phosphorylation, which modulates protein activity and protein–protein interactions, activation of transcription factors and production of antimicrobial proteins and peptides. In addition, it is important to mention a family of peptides, the defensins, which are common to mammals, insects and plants, and have wide spectra of activity directed against bacteria, fungi and viruses [9, 10]. The intriguing hypothesis that emerges from the discovery of similar components and strategies of innate immunity in different organisms is that a group of proteins arose in a putative common ancestor of animals and plants, and has served to protect against infection throughout evolution (figure 1).

In recent years, many insights have been gained about the molecular mechanisms that allow resistant tomato plants to defeat infection of the bacterial pathogen *P. syringae* pv. *tomato*. This resistance phenomenon is mediated by the IRAK- and Pelle-homolog Pto kinase, which serves as a sensor of pathogen invasion and as a trigger of signaling pathways leading to disease resistance. In this article we review the molecular and biochemical properties of Pto and of additional components of the Pto pathway, which represents a paradigm for innate immunity in plants.

## 2. Speck disease resistance in tomato

Bacterial speck in tomato is caused by the pathogen *P. syringae* pv. *tomato*. Typical symptoms of this disease, which affects the aerial parts of the plant, are necrotic lesions surrounded by a chlorotic halo (figure 2A). Early symptoms are followed by death of leaves and plant defoliation. Resistance to bacterial speck was originally derived from the wild tomato species *Lycopersicon pimpinellifolium* and introgressed into the cultivated species of tomato *Lycopersicon esculentum*. Plants carrying the resistance trait inoculated with *P. syringae* are completely free of disease symptoms compared to susceptible plants (figure 2A and 2B). The genetic basis of speck disease resistance conforms to a classical gene-for-gene interaction, according to which resistance occurs only when an *R* gene is expressed in the plant and the corresponding *avr* gene is present in the pathogen [11].

### 2.1. The Pto gene

The *Pto* gene, which confers resistance to bacterial speck and is responsible for the resistance trait of *L. pimpinellifolium* to *P. syringae*, was isolated by map-based cloning [8], and its introduction into a susceptible tomato
cultivar resulted in a marked increase in resistance to avrPto-expressing strains of *P. syringae*. When plants expressing *Pto* are infected by AvrPto-containing *P. syringae*, they rapidly develop a typical immune reaction (HR) which is manifested by rapid tissue collapse localized at the site of infection. The ability of the *Pto* gene product to trigger defense mechanisms was further confirmed by the finding that overexpression of *Pto* in transgenic tomato plants confers broad resistance and results in the activation of various defense responses [12].

The *Pto* gene is part of a small gene family, which consists of five members clustered on tomato chromosome five. Interestingly, one member of this family is the *Fen* gene, which encodes a serine/threonine protein kinase that mediates a hypersensitive-like response in tomato plants treated with the organophosphorous insecticide fenthion [13]. There is evidence that functional *Pto* homologues exist in other plant species, including soybean and tobacco [14, 15]. Furthermore, *Pto*-related sequences have been detected in a wide spectrum of plant species, ranging from monocotyledons to dicotyledons [8]. Thus, a signaling pathway involving a *Pto*-like gene may be widely conserved in the plant kingdom.

The *Pto* gene encodes a functional serine/threonine protein kinase [16], which autophosphorylates in vitro by an intramolecular mechanism [17]. A three-dimensional model for the structure of the *Pto* catalytic domain was obtained by homology modeling using as templates protein kinases of known structure (figure 3) [18]. The predicted *Pto* molecule resembles the typical structure of a protein kinase catalytic domain, consisting of a small lobe, which is involved in ATP binding and orientation, and of a large lobe which provides sites for substrate recognition and catalysis [19]. In a biochemical study, eight sites of autophosphorylation were identified in the *Pto* molecule [18]. Among them, Ser-17 and Thr-38, which are the major autophosphorylation sites, are located outside the kinase catalytic domain in close proximity to the *Pto* small lobe and to the ATP binding site.

Six additional autophosphorylation sites are located in the *Pto* kinase catalytic domain. Remarkably, sites Thr-190, Thr-195, Ser-198 and Thr-199 are within the *Pto* region corresponding to the segment defined as the kinase activation domain, which in many protein kinases is phosphorylated and involved in the regulation of kinase activity (figure 3) [20].

An intracellular localization has been proposed for the Pto kinase based on the observation that it does not contain obvious extracellular or membrane-spanning domains. A putative myristylation motif is present at its N-terminus, and may be utilized by the cell to recruit Pto to the plasma membrane. However, the insertion of a mutation in the invariant glycine of the Pto myristylation site did not affect Pto-mediated speck disease resistance [21].

2.2. The *AvrPto* gene and secretion of its product by the bacterial type III secretion system

The presence of the *avrPto* gene in the bacterium is required for an incompatible (resistant) interaction with *Pto*-expressing tomato plants. The *avrPto* gene was cloned from *P. syringae*, and encodes a small (18.3 kDa) and mostly hydrophilic protein with no homology to protein sequences in databases [22]. The introduction of the cloned *avrPto* into normally virulent strains of *P. syringae* converts them into avirulent strains [14]. The avirulence function of *avrPto* and other *avr* genes has been shown to be dependent on pathogen genes designated *hrp* (hypersensitive response and pathogenicity), which encode proteins of the type III secretion system (also called the *hrp* secretion pathway or system) [23]. Several Gram-negative pathogenic bacteria utilize the type III secretion system to deliver into the host cell effector proteins that modulate host cellular functions [24]. This secretory machinery is present in plant and animal pathogenic bacteria and is evolutionarily related to the flagellar apparatus. In line with a requirement of *hrp* genes for the *avrPto* avirulence function, the promoter region of *avrPto* has been found to contain a regulatory sequence present upstream of *avr* and *hrp* genes referred to as *hrp* box motif [22]. Moreover, the AvrPto protein has been shown to be secreted by the *hrp* system of the plant pathogens *P. syringae* and *Erwinia chrysanthemi* [25, 26], and also by the heterologous type III secretion system of the mammalian pathogen *Yersinia* [27]. Targeting signals for secretion by the *Yersinia* and *Erwinia* type III machinery of the avirulence proteins AvrPto and AvrB of *P. syringae* are located at the 3′ end of the mRNA, as is the case for *Yersinia* yopE and yopQ [27].
Although the AvrPto protein has been shown to be secreted by the type III secretion apparatus, there is no direct evidence to date for its translocation from the bacterium to the plant cytoplasm. However, transient expression of AvrPto in plant cells bypasses the requirement of pathogen functions mediated by hrp genes and induces a Pto-dependent HR, strongly suggesting that AvrPto acts inside the plant cell [28, 29]. The localization of AvrPto within the plant cell was studied in transgenic tobacco plants using a tetracycline-inducible gene expression system (X. Tang, Kansas State University; personal communication). In these plants AvrPto was found to be exclusively associated with membranes. Interestingly, AvrPto contains at the N-terminus a myristylation motif, and a mutation in this motif completely abolished the avirulence function of AvrPto in tomato and tobacco plants (X. Tang, personal communication). Taken together, this evidence suggests that during pathogenesis AvrPto is secreted inside the plant cell by the bacterial type III secretion system, which recognizes the avrPto mRNA targeting signal. Within the plant cell the association of AvrPto with membranes allows the protein to be functional as an avirulence determinant.

3. Pto–AvrPto recognition: an intracellular receptor–ligand interaction

In plant disease resistance, the molecular basis of gene-for-gene interactions is thought to reside in the specific recognition between a receptor encoded by the plant $R$ gene and an elicitor molecule produced by the pathogen. In support of this receptor–ligand model, a physical interaction was detected between the Pto kinase and the AvrPto protein, by using the yeast two-hybrid system [28, 29]. The Pto–AvrPto interaction detected in yeast was strictly correlated to the expression of disease resistance in plants. Deletion of AvrPto sequences required for Pto interaction impaired the ability of AvrPto to elicit defense responses. Conversely, removal of AvrPto portions dispensable for Pto interaction did not affect resistance [29].

Pto autophosphorylation activity appears to be required for Pto–AvrPto physical interaction and for the elicitation of the HR. In fact, a mutation at Thr-38, the major site of Pto autophosphorylation, abolishes this receptor–ligand interaction and the development of the HR [18]. In addition, several mutations at two residues, Lys-69 and Asp-164, which are required in all protein kinases for ATP binding and catalysis, respectively [30], interfere with Pto kinase activity and the capability of Pto to interact with AvrPto [28, 29, 31]. In this context, the only exception is represented by the substitution of Asp-164 with Asn, which disrupted Pto kinase activity but not the AvrPto–Pto interaction [31]. It is possible that the structure of Pto (D164N) mimics the conformation of autophosphorylated Pto. Remarkably, Thr-38, which is the main Pto autophosphorylation site and is required for Pto function, is conserved in IRAK and Pelle kinases [18]. It will be interesting to determine if Thr residues, corresponding in the human IRAK and the Drosophila Pelle kinases to Pto Thr-38, are also major autophosphorylation sites and if they are required for regulatory functions.

A domain swapping analysis between Pto and the closely related Fen protein kinase has identified a region in the Pto kinase activation domain which is determinant for Pto–AvrPto interaction and specificity [28, 29]. Within this region, Thr-204 is required for the specific recognition of AvrPto, as tested in the yeast two-hybrid system, and for the elicitation of the HR in plants (figure 3) [32]. Moreover, introduction of a Thr at the amino acid location corresponding to Pto Thr-204 confers to Fen the ability to interact with AvrPto and to induce an HR. It is interesting to note that the Pelle and IRAK kinases also have a Thr conserved at the position corresponding to Pto Thr-204.

AvrPto residues that determine specificity for the interaction with Pto were identified by testing AvrPto molecules randomly mutagenized at single amino acids for their interaction with Pto in a yeast two-hybrid system (X. Tang, personal communication). This analysis revealed that a stretch of amino acid in the central part of the AvrPto molecule, from residue 94 to 99, is required for the interaction with Pto and for disease resistance in tomato but not in tobacco plants, which contain a functional Pto homolog. Conversely, a cluster of amino acids located at the C-terminus of the AvrPto molecule is required for the development of disease resistance in tobacco but not in tomato plants. Although a Pto homolog from tobacco has not been isolated yet, this evidence suggests that distinct motifs in the AvrPto molecule determine specificity for tomato and tobacco Pto.

The location within the cell of the interaction between Pto and AvrPto has yet to be established. However, the requirement of the AvrPto myristylation motif for AvrPto avirulence function raises the possibility that the Pto–AvrPto interaction takes place in association with membranes, and it is mediated either by myristylation or adaptor proteins.

4. Molecular mechanisms involved in Pto activation

The physical interaction between the Pto kinase and AvrPto provides a molecular explanation for gene-for-gene specificity in plant disease resistance. However, it remains to be elucidated how the formation of the AvrPto–Pto complex results in Pto activation. It is possible that the binding of AvrPto to Pto causes a conformational change in the structure of the Pto activation domain, which results in induction of Pto activity. In support of this model, it was found that the mutant Pto (Y207D), which has a substitution in a residue of the kinase activation domain, mediates the elicitation of the HR in the absence of AvrPto [31]. Interestingly, Pto (Y207D) is able to induce an HR only in the presence of an additional gene, Prf, which is also required for bacterial speck disease resistance and the related phenotype of fenithion sensitivity [33].

Autophosphorylation has been implicated in the mechanism of Pto activation [18]. In fact, mutation of the Pto autophosphorylation site Ser-198, which is located in the activation domain of the molecule, interferes with the elicitation of the HR, but not with the AvrPto–Pto interac-
dication. The activation domain is the target of regulatory autophosphorylation in several serine/threonine and tyrosine kinases [20], raising the possibility that the interaction with AvrPto causes a conformational change in the Pto molecule, which exposes the regulatory site Ser-198 to autophosphorylation. Phosphorylation at Ser-198 may be involved in induction of Pto kinase activity or in the association of Pto with downstream effectors. In line with the latter hypothesis, a mutation at Ser-198 was found to alter the physical interaction of Pto with the serine/threonine kinase Pti1, and with the two proteins of unknown function Pti3 and Pti10 [18].

An additional possibility for Pto activation is that its interaction with AvrPto allows it to take part in a multisubunit receptor complex with other plant molecules. The Prf protein is a possible candidate to serve as an adaptor molecule in the formation of a complex involving Pto, AvrPto and additional proteins. Prf, which is required for Pto-mediated disease resistance, is a large molecule (209.7 kDa), and contains leucine-rich repeats that for other proteins have been shown to mediate protein–protein interactions [33]. The effect of complex formation could be either activation of Pto kinase activity or its recruitment to a target cellular compartment.

5. Signal transduction originating from the AvrPto–Pto recognition event

The Pto–AvrPto recognition event is postulated to activate the Pto kinase and induce phosphorylation of downstream components in signaling pathways leading to defense responses (figure 4). Downstream effectors, which physically interact with Pto and represent putative targets for its phosphorylation, were isolated by using a yeast two-hybrid system [34, 35]. Among them and of particular significance are the protein kinase Pti1, which is involved in the elicitation of the HR, and the transcription factors Pti4, Pti5 and Pti6, which are thought to drive the expression of pathogenesis-related genes.

5.1. Pto phosphorylation of Pti1 and the hypersensitive response

Pti1 is a cytoplasmic serine/threonine protein kinase, and its role in Pto-mediated disease resistance was assessed in transgenic tobacco plants [34]. In these plants, overexpression of Pti1 enhanced the HR in leaves challenged with Pseudomonas syringae pv. tabaci expressing the avrPto gene. In vitro analysis revealed that Pti1 is able to autophosphorylate via an intramolecular mechanism, and it is specifically phosphorylated by Pto [17, 34]. Taken together, this evidence suggests that Pti1 acts downstream of Pto in a phosphorylation cascade that triggers the hypersensitive response.

A biochemical approach was used to characterize further the molecular mechanisms involved in the interaction between the Pto and Pti1 protein kinases [17, 18, 35]. Pto was found to phosphorylate Pti1 at one major and several minor sites with a \( k_a \) of 4.1 \( \mu \)M and a \( V_{\text{max}} \) of 0.55 nmol/min/mg. The major site phosphorylated by Pto in Pti1 was identified as Thr-223, which is located in the Pti1 kinase activation domain. Mutational analysis revealed that this site is also the major Pti1 autophosphorylation site.

Both Pto and Pti1 phosphorylation sites play roles in the physical interaction between Pto and Pti1, as observed in a yeast two-hybrid system [18, 34, 36]. The Pto–Pti1 interaction requires Pto kinase activity [34], and it is affected by mutations at Pto autophosphorylation sites. In fact, a mutation at Pto Ser-198 enhanced the association between the two proteins, while mutations at Thr-199 and Thr-288 decreased the interaction [18]. In addition, Pti1 kinase activity is dispensable for its interaction with Pto, which still requires the main Pti1 phosphorylation site Thr-233 [36].

The dynamics of Pto and Pti1 physical interaction and phosphorylation in vivo remain to be established. Pto and Pti1 could form a transient complex that is released upon Pti1 phosphorylation or more likely a durable complex stabilized by phosphorylation. It will be important also to assess the physiological relevance of Pti1 phosphorylation by Pto in vivo, and how phosphorylation of Pti1 Thr-233 affects Pti1 kinase activity in the onset of speck disease resistance. In addition, physiological substrates for Pti1 phosphorylation have yet to be identified.
5.2. Pto interaction with Pti4/5/6 and expression of PR genes

A major target of signal transduction pathways leading to defense responses in plants is the transcriptional activation of defense-related genes [37]. In speck disease resistance of tomato and tobacco, for example, transcripts of a set of defense genes encoding PR proteins accumulate earlier during resistant interactions than in susceptible interactions [35, 38]. In the Pto signaling pathway, a possible bridge linking signal recognition to activation of defense genes is represented by the transcription factors Pti4, Pti5 and Pti6 (Pti4/5/6). The Pti4/5/6 proteins were found to physically interact with Pto in a yeast two-hybrid screen [35]. Interestingly, Pto autophosphorylation was required for the interaction of Pto with Pti4/5/6, and mutations at specific Pto autophosphorylation sites altered the Pto–Pti4 interaction [18]. Typical structures of transcription factors, which are present in the amino acid sequence of Pti4/5/6, are a central DNA-binding domain rich in basic residues, a region of acidic residues possibly involved in transcriptional activation, and nuclear localization sequences. Interestingly, Pti4/5/6 are able to bind in a gel-shift assay a cis-acting element required for ethylene responsiveness and present in promoters of basic-type PR genes (GCC box) [35, 39]. In addition, transcription of Pti4 and Pti5 was found to be induced in both resistant and susceptible interactions of tomato with P. syringae [40]. Taken together, the interaction between Pto and Pti4/5/6, and the binding of Pti4/5/6 to the GCC box, suggest that Pto may regulate PR gene expression by the direct activation of these transcription factors.

The mechanisms that allow Pto to interact with and possibly activate Pti4/5/6 by Pto are still unclear. Recently Pti4 has been found to be phosphorylated at multiple sites by Pto in a kinase assay in vitro [39]. A phosphorylation event may result in the activation of the transcription factors Pti4/5/6, by regulating their binding activity or transcriptional potential. Interestingly, phosphorylation of Pti4 by Pto in vitro enhanced the ability of this transcription factor to bind a GCC box element [39]. Phosphorylation may also play a critical role in localizing Pti4/5/6 to the nucleus. To distinguish between these different mechanisms of activation, it will be necessary to elucidate in vivo the effect of phosphorylation on Pti4/5/6 activity and localization during pathogen challenge.

6. Concluding remarks

The Pto pathway in tomato represents one of the best-characterized examples of innate immunity in plants. As summarized in the model presented in figure 4, an early recognition event between Pto and the bacterial protein AvrPto triggers the rapid activation of several plant defense responses that in concert lead to disease resistance. A better understanding of the molecular strategies that plants utilize to defend themselves against pathogens will allow improving disease resistance in economically important crops. In addition, as suggested by the structural and functional homologies that are preserved in components of innate immunity systems in plants, animal and insects, further characterization of plant responses to pathogens will also contribute to our understanding of innate immunity in different multicellular organisms.

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